

Caution! Analyze transcripts from conditional knockout alleles

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Abstract A common strategy for conditional knockout alleles is to “flox” (flank with *loxP* sites) a 5' exon within the target gene. Typically, the floxed exon does not contain a unit number of codons so that the *Cre*-mediated recombination event yields a frameshift and a null allele. Documenting recombination within the genomic DNA is often regarded as sufficient proof of a frameshift, and the analysis of transcripts is neglected. We evaluated a previously reported conditional knockout allele for the β -subunit of protein farnesyltransferase. The recombination event in that allele—the excision of exon 3—was predicted to yield a frameshift. However, following the excision of exon 3, exon 4 was skipped by the mRNA splicing machinery, and the predominant transcript from the mutant allele lacked exon 3 and

exon 4 sequences. The “ Δ exon 3–4 transcript” does not contain a frameshift but rather is predicted to encode a protein with a short in-frame deletion. This represents a significant concern when studying an enzyme, since an enzyme with partial function could lead to erroneous conclusions. With thousands of new conditional knockout alleles under construction within mouse mutagenesis consortiums, the protein farnesyltransferase allele holds an important lesson—to characterize knockout alleles at both the DNA and RNA levels.

Keywords Protein prenylation · Farnesylation · Prelamin A · HDJ-2 · Knockout mice

Abbreviations

Fntb Farnesyltransferase
FTI Farnesyltransferase inhibitor
MEF Mouse embryonic fibroblast

Introduction

In preparing a conditional knockout allele, *loxP* sites are typically inserted into different introns of a target gene (“floxed” an exon or exons) (Kühn et al. 1995; Rajewsky et al. 1996; Schwenk et al. 1995). With the expression of *Cre* recombinase, the exon(s) of the gene is removed, resulting in a knockout allele. Typically, care is taken to “flox” an exon without a

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unit number of codons (i.e., an exon where the number of nucleotides in the exon is not evenly divisible by three) so that the recombination event yields a frameshift. A frameshift is important for creating a null allele (Hasty et al. 2000). Other considerations relevant to producing a null allele have been reviewed by Hasty et al. (2000).

A conditional knockout allele for *Fntb*, the gene encoding the β -subunit of protein farnesyltransferase (FTase), was reported by Mijimolle et al. (2005). FTase catalyzes the farnesylation (covalent attachment of a 15-carbon isoprenyl lipid) of proteins carrying a carboxyl-terminal *CaaX* motif (Casey and Seabra 1996). In Mijimolle's *Fntb* conditional knockout allele (Mijimolle et al. 2005), exon 3 (73 bp in length) was flanked by *loxP* sites. As expected, the expression of *Cre* recombinase excised exon 3, and that recombination event was well characterized at the genomic DNA level (Mijimolle et al. 2005). The excision of exon 3 was predicted by Mijimolle et al. (2005) to yield a frameshift and a null allele, but transcripts from the allele were not analyzed.

Without question, the *Cre*-mediated excision of exon 3 reduced FTase activity. Germline homozygosity for the recombined allele prevented normal embryonic development, and fibroblasts homozygous for the mutant allele had reduced FTase activity (Mijimolle et al. 2005). On the other hand, several features of Mijimolle's study (Mijimolle et al. 2005) were unexpected. For example, in homozygous knockout fibroblasts, H-RAS remained associated with membrane fractions (Mijimolle et al. 2005). That was peculiar because the association of H-RAS with membranes is utterly dependent on protein farnesylation (Berzat et al. 2005, 2006; Chenette et al. 2005; Takahashi et al. 2005). The prenylation of another farnesylated protein, HDJ-2, was only partially blocked in their homozygous *Fntb*-deficient fibroblasts (Mijimolle et al. 2005). Also, the *Fntb* knockout had no effect on tumorigenesis, while FTase inhibitor drugs retard tumor growth (Barrington et al. 1998; Omer et al. 2000).

In this study, we examined the *Fntb* allele generated by Mijimolle et al. (2005). We found that the predominant transcript produced by the mutant allele did not contain a frameshift but rather encoded a protein with a short in-frame deletion.

Materials and methods

Genetically modified mice

Mice harboring an *Fntb* conditional knockout allele (*Fntb*^{lox}) and a fully recombined knockout allele (*Fntb*^Δ) (Mijimolle et al. 2005) were obtained from the Fundación Centro Nacional De Investigaciones Oncológicas Carlos III. Mice were genotyped by PCR with oligonucleotide primers 5'-CTGGAGGCTGGGAAGCTGGA-3' and 5'-GCCTAAGAGCCGTGTGGGGGT-3'. The wild-type allele yielded a 1,940-bp fragment; the *Fntb*^{lox} allele, a 907-bp fragment; the *Fntb*^Δ allele, a 557-bp fragment.

Fibroblasts from *Fntb*^{lox/Δ} embryos were obtained as previously described (Todaro and Green 1963). Mouse embryonic fibroblasts (MEFs) (80% confluent) grown on 6-well plates were infected with *Cre* adenovirus (20 μ l/well, 3×10^{10} pfu/ml, Gene Transfer Vector Core, University of Iowa), and cells extracts were prepared 5 days later. In some experiments, cells were treated with *Cre* adenovirus only once. A single treatment with *Cre* adenovirus generally did not lead to 100% complete recombination; however, those experiments were nevertheless useful because they allowed us to analyze *Cre*-recombination events.

In other experiments, *Cre* adenovirus treatment was repeated three times, so as to yield complete recombination. Before harvesting the latter cells, genotyping was performed to make sure that the *Fntb*^{lox/Δ} cells had been converted to the *Fntb*^{Δ/Δ} genotype. In some experiments, fibroblasts were treated with a protein farnesyltransferase inhibitor (FTI) (ABT-100, 5 μ M) (Ferguson et al. 2005).

RT-PCR reactions

RT-PCR products were obtained from *Fntb*^{lox} and *Fntb*^Δ alleles with exon1/exon5, exon1/exon8, and exon1/exon4 primer sets. Exon 1 forward primer: 5'-TACAGCGCTCGCAGCTCTCC-3'; exon 5 reverse primer: 5'-CATTGACGGCTGCATAAGTG-3'; exon 8 reverse primer: 5'-GCCAAGCCACAGAAGGTGTA-3'; exon 4 reverse primer: 5'-CTGCTACTGGATCCTGCAC A-3'. Total RNA was extracted from MEFs or tissues with the RNeasy Mini kit (Qiagen); reverse transcriptase reactions were carried out with 500 ng of RNA that had been treated with DNase (DNA-Free, Ambion),

Superscript III (Invitrogen), and a mixture of oligo-dT (Invitrogen) and random primers (Invitrogen). The thermal cycling conditions were as follows: 95°C for 3 min, 95°C for 15 s, 60°C for 30 s, and 72°C for 15 s, for a total of 26 cycles.

Western blots

Cell extracts were electrophoresed on 4–12% gradient polyacrylamide Bis–Tris gels (Invitrogen), and the proteins were electrophoretically transferred to a nitrocellulose membrane for western blotting. The antibody dilutions were 1:400 for anti-lamin A/C goat IgG (sc-6215, Santa Cruz Biotechnology), 1:500 for anti-HDJ-2 mouse IgG (NeoMarkers), and 1:1000 for anti-actin goat IgG (sc-1616, Santa Cruz Biotechnology). After washing the membrane, the blots were incubated with secondary antibodies (1:5000 IRDye 700 anti-goat IgG antibody and 1:5000 IRDye 800 anti-mouse IgG antibody). Signals were detected with the Odyssey infrared imaging system (LI-COR Biosciences).

Results and discussion

We generated *Fntb*^{lox/Δ} MEFs and incubated them with *Cre* adenovirus (Anton and Graham 1995) or an adenovirus encoding β-galactosidase. As expected, the floxed segment of DNA (spanning exon 3) was removed by *Cre* recombinase (Fig. 1a, b). The location of the *loxP* sites in the *Fntb*^{lox} allele was documented by DNA sequencing. As noted earlier, the excision of exon 3 was predicted to yield a

frameshift (Mijimolle et al. 2005). To characterize the transcripts from the mutant allele, we performed RT-PCR reactions spanning sequences encoded by exons 1–5 and exons 1–8 (Fig. 2a–c). Following the excision of exon 3 from the genomic DNA, exon 4 was skipped by the mRNA splicing machinery. Thus, the predominant transcript from the *Fntb*^Δ allele in *Fntb*^{lox/Δ} MEFs lacked exon 3 and exon 4 sequences (Fig. 2b, c). After a single round of *Cre* adenovirus infection, the amount of “Δexon 3–4 transcript” was substantial, but some non-recombined (wild-type) transcripts remained (Fig. 2b). To achieve complete recombination, we treated *Fntb*^{lox/Δ} MEFs with three rounds of *Cre* adenovirus (Fig. 2c). In the latter cells, none of the wild-type *Fntb* transcript remained.

The identity of the RT-PCR product corresponding to the “Δexon 3–4 transcript” was confirmed by DNA sequencing (Fig. 2d). The Δexon 3–4 transcript was the predominant transcript from the *Fntb*^Δ allele within the tissues of *Fntb*^{lox/Δ} mice (Fig. 2e). The Δexon 3–4 transcript is predicted to encode a protein with an in-frame deletion of amino acids 70–124.

As judged by ethidium bromide-stained agarose gels of RT-PCR reactions, the levels of the Δexon 3–4 transcript in *Fntb*^{lox/Δ} MEFs and mouse tissues were comparable to those of the wild-type *Fntb* transcript (Fig. 2b, c, e). Finding high levels of the Δexon 3–4 transcript was not surprising because the Δexon 3–4 transcript lacks a premature stop codon and would not be subject to nonsense-mediated mRNA decay.

Trace amounts of a Δexon 3 RT-PCR product could be detected between the wild-type and Δexon 3–4 RT-PCR products (Fig. 2b, e). The identity of the Δexon 3 RT-PCR product was confirmed by DNA

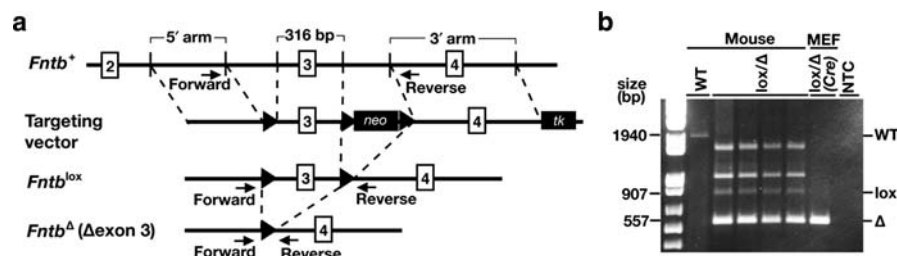


Fig. 1 A conditional knockout allele for *Fntb*, the gene encoding the β-subunit of FTase. **a** Strategy of Mijimolle et al. 2005 to create a conditional *Fntb* knockout allele. *Cre*-mediated excision of the floxed DNA segment removes exon 3, a deletion that was predicted to yield a frameshift. **b** PCR strategy to genotype mice or mouse embryonic fibroblasts.

The PCR product yielded a 1,940-bp fragment from the wild-type *Fntb* allele, a 907-bp fragment from the *Fntb*^{lox} allele, and a 557-bp fragment from the *Fntb*^Δ allele. The two extra bands, with apparent lengths of ~1,100 and 1,800 bp, are heteroduplexes

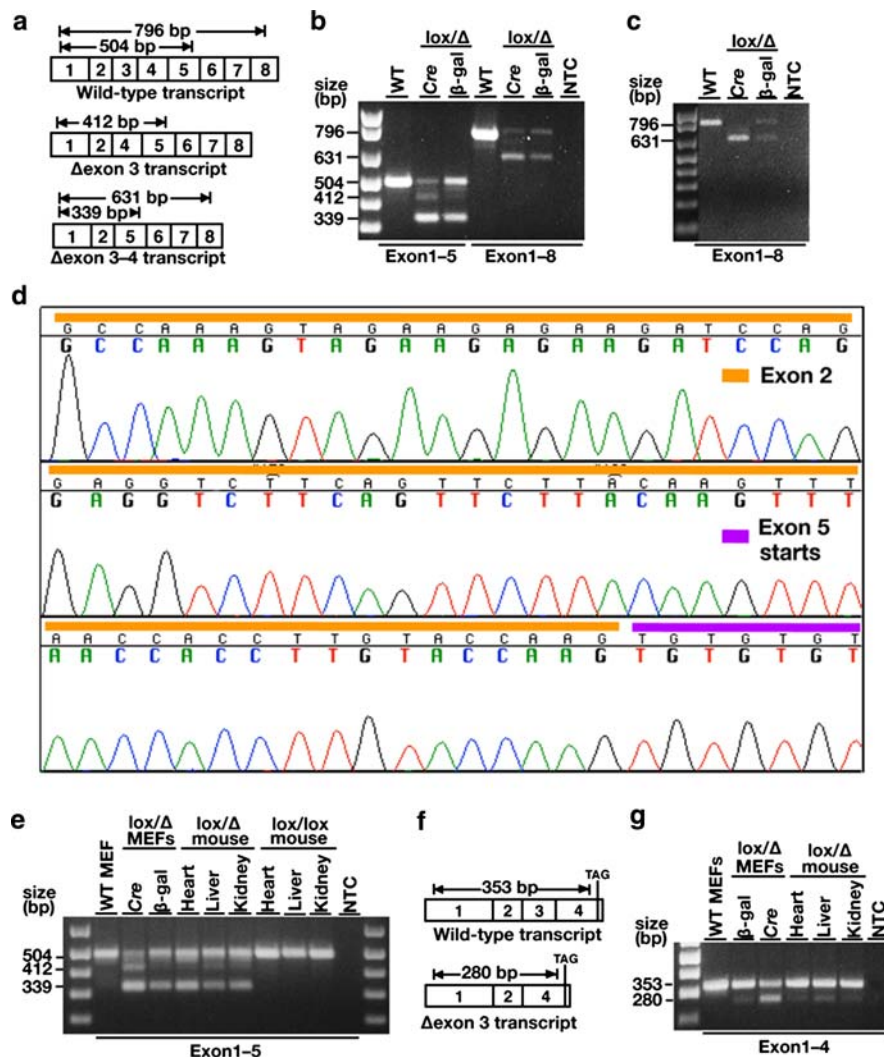


Fig. 2 Characterizing transcripts from the *Fntb*^Δ allele. **a** Schematic illustrating RT-PCR products obtained from *Fntb*^{lox} and *Fntb*^Δ alleles with an exon1/exon5 primer set and an exon1/exon8 primer set. **b** Ethidium bromide-stained 1.5% agarose gel showing the RT-PCR products generated from RNA prepared from wild-type (WT) MEFs, β-galactosidase adenovirus-treated *Fntb*^{lox/Δ} MEFs, and *Cre* adenovirus-treated *Fntb*^{lox/Δ} MEFs. With the exon1/exon5 primer set, the fainter amplicon between the 504-bp wild-type *Fntb* RT-PCR product and the 339-bp Δexon 3–4 RT-PCR product is the 412-bp Δexon 3 RT-PCR product. The numbers on the left indicate sizes of the RT-PCR products. **c** Ethidium bromide-stained 1.5% agarose gel showing the RT-PCR products (with the exon1/exon8 primer set) with RNA from wild-type (WT) MEFs and *Fntb*^{lox/Δ} MEFs that had been treated three times over 7 days with β-galactosidase adenovirus and *Cre* adenovirus. The RT-PCR reaction yields a 796-bp wild-type band and a 631-bp “Δexon 3–4” product. The numbers on the left indicate sizes of the RT-PCR products. **d** DNA sequencing chromatogram showing the sequence of the Δexon 3–4 RT-PCR product. The DNA fragment that was

sequenced and purified from the agarose gel with the QIAquick Gel Extraction Kit (Qiagen). **e** Ethidium bromide-stained agarose gel showing the RT-PCR products generated from the exon1/exon5 primer set and RNA from *Fntb*^{lox/Δ} MEFs, the tissues of an *Fntb*^{lox/Δ} mouse, and tissues of an *Fntb*^{lox/lox} mouse. The predominant products were the 504-bp wild-type *Fntb* RT-PCR product and the 339-bp Δexon 3–4 RT-PCR product. The numbers on the left indicate sizes of the RT-PCR products. **f** Schematic illustrating RT-PCR products obtained from *Fntb*^{lox} and *Fntb*^Δ alleles with an exon1/exon4 primer set. **g** Ethidium bromide-stained agarose gel showing the RT-PCR products generated from the exon1/exon4 primer set and RNA prepared from WT MEFs, β-galactosidase adenovirus-treated *Fntb*^{lox/Δ} MEFs, *Cre* adenovirus-treated *Fntb*^{lox/Δ} MEFs, and tissues of an *Fntb*^{lox/Δ} mouse. A strong 353-bp WT RT-PCR product and a fainter 280-bp Δexon 3 RT-PCR product could be detected in tissues of an *Fntb*^{lox/Δ} mouse and in *Fntb*^{lox/Δ} MEFs. After treating the cells with *Cre* adenovirus, the Δexon 3 RT-PCR product was more intense. The numbers on the left indicate sizes of the RT-PCR products.

sequencing (not shown). The Δ exon 3 transcript could also be detected by RT-PCR with an exon1/exon4 primer set (this primer set cannot detect the Δ exon 3–4 transcript) (Fig. 2f, g). The level of the Δ exon 3 transcript, relative to the wild-type transcript, was low (Fig. 2b, e).

Prelamin A and HDJ-2 are both *CaaX* proteins that are farnesylated by FTase. In *Cre* adenovirus-treated *Fntb*^{lox/ Δ} MEFs (three rounds of infection), we found that HDJ-2 prenylation was blocked by only 50% (Fig. 3a). These results were essentially identical to the findings reported earlier by Mijimolle et al. (2005); they had produced *Fntb* ^{Δ/Δ} fibroblasts from a single cell clone and found that only ~50% of HDJ-2 prenylation was blocked. In control experiments with wild-type fibroblasts, we found that a pharmacologic inhibitor of FTase blocked almost all HDJ-2 farnesylation (Fig. 3a).

We also found small amounts of prelamins A accumulation in these cells, indicating a partial blockade of lamin A biogenesis (Fig. 3b). Normally, prelamins A is efficiently converted to mature lamin A and prelamins A is virtually undetectable in cells. In the absence of farnesylation, prelamins A cannot be converted to mature lamin A and nonfarnesylated prelamins A accumulates in cells. Interestingly, the amount of prelamins A in *Cre* adenovirus-treated *Fntb*^{lox/ Δ} MEFs was small but increased when the cells were treated with an FTI (Fig. 3b). The existence

of nonfarnesylated HDJ-2 and prelamins A in *Cre* adenovirus-treated *Fntb*^{lox/ Δ} MEFs provided unequivocal evidence for markedly reduced FTase activity. On the other hand, the effects of the *Fntb* gene inactivation on HDJ-2 prenylation and lamin A biogenesis were less than we would have expected with a complete *Fntb* knockout. Also, we observed greater inhibition of HDJ-2 prenylation when *Cre* adenovirus-treated *Fntb*^{lox/ Δ} MEFs were treated with an FTase inhibitor (Toth et al. 2005; Yang et al. 2005).

At this point, we do not know if a mutant β -chain lacking amino acids 70–124 would be able to associate with the α -chain and yield an FTase complex with residual enzymatic activity. However, this possibility is not farfetched; deletions of >100 amino acids from the amino terminus of the β -subunit of yeast FTase do not prevent association with the α -chain (Urano et al. 2000). We also do not know if the internally truncated β -chain would yield a prenyltransferase with altered specificities. Addressing these issues with biochemical studies would be extremely challenging. In fact, we believe that biochemical experiments to *fully exclude* trace amounts of residual FTase activity from Mijimolle's *Fntb* ^{Δ} allele (and biochemical experiments to *unequivocally prove* that the prenylation in *Fntb* ^{Δ/Δ} cells is due to geranylgeranyltransferase, type I) would be virtually impossible and ultimately would yield murky results.

In some knockout experiments, a 99.5%-complete knockout might be entirely satisfactory. However, when the goal is to investigate the physiologic importance of FTase, a “leaky” knockout allele is suboptimal. When faced with an in-frame deletion and the possibility of an enzyme with partial function, one should strongly consider generating a new conditional knockout allele.

In constructing their conditional *Fntb* knockout allele, Mijimolle et al. (2005) reported that they had deleted 967 bp of intron 2 and 297 bp of intron 3. Based on their paper, the intron 2 deletion started 114 bp 5' to the intron 2–exon 3 junction, and the intron 3 deletion started 146 bp 3' to the exon 3–intron 3 junction. These descriptions did not match our DNA sequencing data; the deletions started 155 bp 5' and 88 bp 3' to exon 3. We believe that introducing deletions this close to intron–exon junctions is hazardous, and we would not be surprised if the intron deletions contributed to the aberrant mRNA splicing. In any case, we suspect that the aberrant splicing event

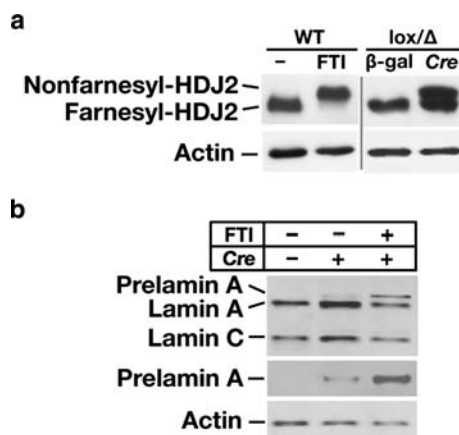


Fig. 3 Western blot analysis of wild-type (WT), *Fntb*^{lox/ Δ} , and *Fntb*^{lox/ Δ} MEFs that had been treated with *Cre* adenovirus or an FTI (ABT-100, 5 μ M). **a** Western blots for HDJ-2; the slower migrating protein is nonfarnesylated HDJ-2. **b** Western blot for lamins A/C and prelamins A in the presence and absence of a specific FTI (ABT-100, 5 μ M)

in Mijimolle's *Fntb* allele would have been overlooked by many laboratories. Conditional knockout alleles are almost always well characterized at the genomic DNA level, and the excision of an exon is widely regarded as proof of a null allele. The transcripts from knockout alleles are often not characterized, even when experimental findings raise the possibility of a leaky allele (Mijimolle et al. 2005).

The NIH has funded the Knockout Mouse Project (KOMP) to create a toolbox of mutant mouse alleles for the biomedical research community (Collins et al. 2007a, b). Thousands of conditional knockout alleles are now being generated. KOMP investigators introduce a *loxP* site and a “splice acceptor–*lacZ* reporter cassette” into one intron and a second *loxP* site into another intron (Collins et al. 2007b). The initial targeting event yields a knockout allele containing a β -galactosidase reporter. However, the *lacZ* cassette (which is flanked by FRT sites) can be excised with *Flp* recombinase, creating a conditional knockout allele in which an exon(s) is flanked by *loxP* sites (i.e., an allele that is structurally similar to the one created by Mijimolle et al. 2005). The studies reported here suggest that caution is warranted with conditional knockout alleles. In experiments with these alleles, whether they are obtained from KOMP or elsewhere, it is important to consider the possibility of unexpected splicing events. This is particularly important for any project where the creation of a true null allele is deemed important.

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